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HIGH-RESOLUTION SCANNING ELECTRON MICROSCOPY OF MILK PRODUCTS: A NEW SAMPLE PREPARATION PROCEDURE

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Abstract

A metal-impregnation technique (tannin-ferrocyanide-osmium tetroxide) and thin metal coating imparted excellent preservation, and electric and thermal properties to milk products for scanning electron microscopy, resulting in high-resolution images. Three nanometer resolution was obtained, with minimal edge effects, charging, thermal drift, and decoration. Samples were fixed in 2% glutaraldehyde, cryoprotected in 70% ethanol, and cryofractured, exposing internal structure. Fat was extracted with Freon 113 and the sample were metal-impregnated with 1.0% osmium tetroxide and 1.5% potassium ferrocyanide, reduced with 1% hydroquinone, dried using the critical-point drying method, and ion beam sputter-coated with 2-5 nm of iridium. Hard cheeses, cream cheese, and yogurt were prepared and imaged using this procedure. Images were recorded at low and high magnifications.

Key Words: Microstructure, cheese, yogurt, high resolution SEM, cryofracturing, potassium ferrocyanide, osmium tetroxide, ion beam sputtering, iridium.

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Introduction

Advances in the development of scanning electron microscopy, particularly the introduction of high-resolution cold-field emission scanning electron microscopes (FESEM), make it possible to distinguish considerably greater detail in samples prepared for microscopy. With most conventional scanning thermionic emitter electron microscopes (SEM), a high accelerating voltage is required for reasonable resolution, which has necessitated thick (20-100 nm) metal coating on non-conductive samples [1, 6], obscuring ultra-fine structural detail such as casein submicelle structure. The thick metal layer is necessary for efficient secondary electron production, and to prevent electron beam damage, charging, and specimen drift. Traditional methods used to prepare milk products for SEM [8] limit high resolution because the thick metal coating obscures ultra-fine detail. At lower magnifications, other problems, such as edge-effect brightness, occur with traditionally prepared samples with a thick metal coating.

Preparation methods that eliminate many of the shortfalls of traditional preparation protocols have not been utilized with milk products [11]. The osmium-thiocarbohydrazide-osmium method (OTO), in which thiocarbohydrazide is used to bind higher quantities of osmium to the specimen, has been used with other types of specimens [8]. However, a layer of metal particles, 3 to 4 nm in diameter, may accumulate on the specimen surface causing decoration (an artificial introduction of new structure) when the OTO method is used [5]. Cheese samples prepared in our laboratory using the OTO method showed inefficient thermal and electrical conductivity, and a thick metal coating was still necessary.

A new metal-impregnation technique, the tannin-ferrocyanide-osmium (TA-F-O) procedure introduced by Hirano *et al.* [5], eliminates the decoration problems of the OTO method. It also imparts superior thermal and electrical conductivity, improves the secondary electron emission, minimizes electron beam irradiation damage, maintains sample rigidity, and preserves ultra-fine structural detail. We have adapted the TA-F-O procedure for the preservation of milk products to produce high-quality and high-resolution images using FESEM. In our previ-

ously published work [9], some problems such as edge-effect brightness, were observed. In this paper, we report an experimental procedure that can be used to overcome such problems and provide a detailed description of our sample preparation and operating procedures.

Materials and Methods

Samples

A variety of milk product samples were examined, including Mozzarella, Swiss, and cream cheeses, and yogurt. Mozzarella cheese was made at Utah State University's Dairy Products Laboratory. Swiss cheese samples were obtained from a Utah cheese manufacturer, and the other samples were purchased as retail items.

Fixation

The samples were cut into blocks (approximately 3 x 3 x 10 mm for cream cheese and yogurt, and 1 x 1 x 10 mm for the others) and were fixed in 2% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in distilled water, at room temperature for 1 hour, followed by 2 to 10 days at 5°C.

Cryofracturing

Prior to cryofixation, the blocks were impregnated with either 70% ethanol or 50% dimethyl sulfoxide (DMSO), or were left in 2% glutaraldehyde. The freezing medium was liquified Freon 22 (-159°C) (Mallinckrodt Inc., Paris, KY). Frozen blocks were transferred to liquid nitrogen and cryofractured perpendicular to their long axes. The cryofractured pieces were thawed in the fluid in which they were originally cryoprotected.

Defatting

Cryofractured samples were defatted with Freon 113 (Mallinckrodt, Inc., Paris, KY) as follows: the blocks were dehydrated with a graded aqueous ethanol (Midwest Grain Co., Westin, MO) series (30%, 50%, 70%, 95%, 100%, 100%, 100%, 10 minutes each) to absolute ethanol and were extracted with Freon 113 in a step-wise fashion (1:3, 1:1, 3:1, pure Freon, 10 minutes each). Samples were stored overnight at 3°C in pure Freon 113, and then rehydrated by reversing the above dehydration schedule.

Metal impregnation and critical point drying

The rehydrated samples were washed for 5 minutes with a 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences, Fort Washington, PA), pH 7.2, and post-fixed for 2 hours with a solution containing 1% OsO_4 (Electron Microscopy Sciences, Fort Washington, PA) and 1.5% $K_4Fe(CN)_6 \cdot 3H_2O$ (Fisher Scientific Co., Fair Lawn, NJ), abbreviated as O-F solution, in the sodium cacodylate buffer. The O-F solution was then replaced with a 2% tannic acid (Mallinckrodt Inc., Paris, KY) solution in the cacodylate buffer and the samples were left to react for 3 hours at 20°C. The tannic acid solution was then replaced with O-F solution for 4 hours, and the O-F solution was subsequently replaced with an aqueous solution of 1% hydroquinone (Mallinckrodt Inc., Paris, KY) and left overnight (18 hours). The samples were then washed with 4 changes of distilled water (10 minutes each), dehydrated in ethanol and impregnated in Freon 113 as described above, and critical-point dried in a 1200 critical point drier (Polaron, Waterford, England) in Freon 13 (DuPont, Antioch, CA).

Specimen mounting and coating

Critical-point dried specimens were mounted on SEM aluminum stubs (Electron Microscope Sciences, Fort Washington, PA) using either a clear finger-nail polish (Cutex Cream Enamel Colorless 07, Chesebrough-Ponds, Inc., Greenwich, CT) or clear finger-nail polish with an overcoat of colloidal silver liquid (Ted Pella Inc., Tustin, CA). Block fragments were mounted with the fractured surface facing upwards.

The specimens were coated with 2 to 4 nm of iridium in an ion beam sputtering system (VCR Group, South San Francisco, CA) at 90° tilt, 75% maximum speed, and 75% maximum rotation. Metal coating thickness was measured with STM-100/MF Thickness/Rate Monitor (Sycon, Syracuse, NY).

Field emission scanning electron microscope imaging

The specimens were examined in a S-4000T FESEM (Hitachi Scientific Instruments, Mountain View, CA) operated at an accelerating voltage between 2 and 10 kV and a specimen current between 0.008 and 0.1 nA. The condenser setting was 10 to 14, and the working distance was 5 to 15 mm. Images were recorded on T-Max 100 film (Kodak, Rochester, NY) at an exposure time of 80 seconds.

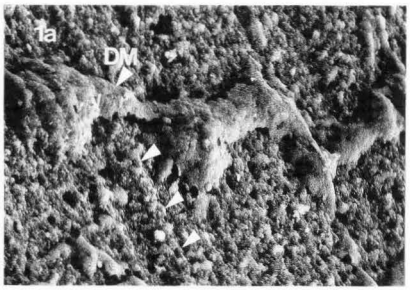
Results

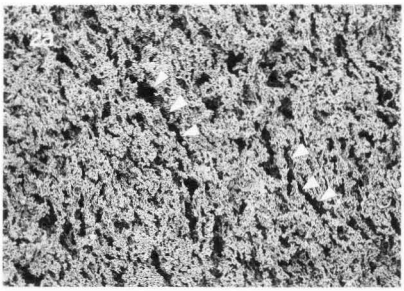
Fixation

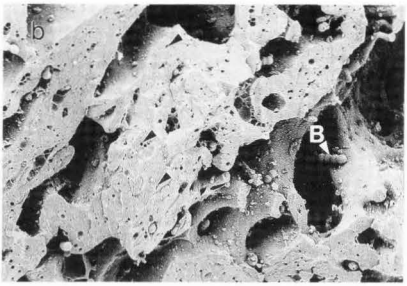
Two-percent glutaraldehyde preserved the protein structures of hard cheeses, soft cheeses, and soft-bodied products like yogurt. Fixation times did not appear to affect image quality from 2 to 10 days. If the fixation had been inadequate, the protein structure would degrade after several days of storage.

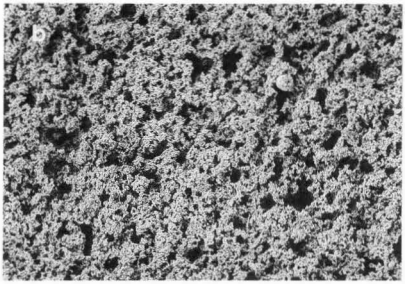
Cryofracturing

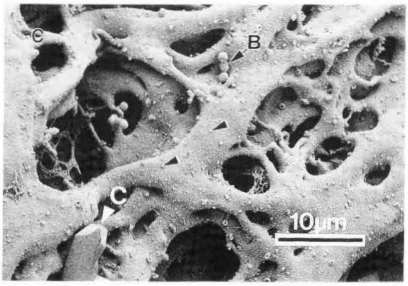
Cut surfaces were distorted, with blade marks and smeared material visible, and had lost much structural information (Fig. 1a). In contrast, the internal structure at and below cryofractured surfaces was well preserved and apparently undistorted (Fig. 1b; and also shown later in Figs. 3a and 3b). The fracture plane remained relatively continuous throughout the specimen. Sometimes, cryofracturing revealed internal voids within the cheese matrix and allowed the matrix to be observed in its native state, thus avoiding smearing of the surface structure caused when a cheese sample was cut at non-cryogenic temperatures. This is well demonstrated with Mozzarella cheese, cut at non-cryogenic temperature (Fig. 1a) as compared to cryofractured cheese (Fig. 1c). The surface of the protein fibers in Figure 1a is covered with a particulate material not seen on the cryofractured











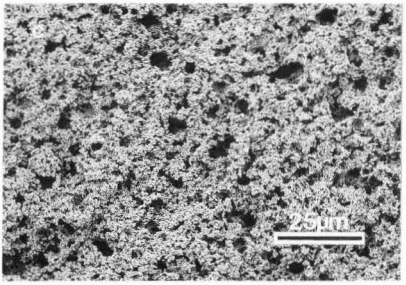
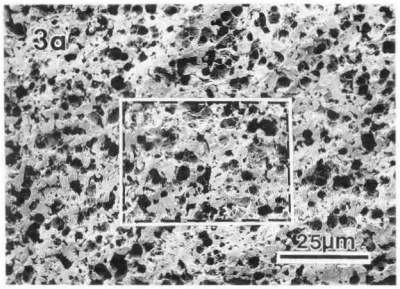
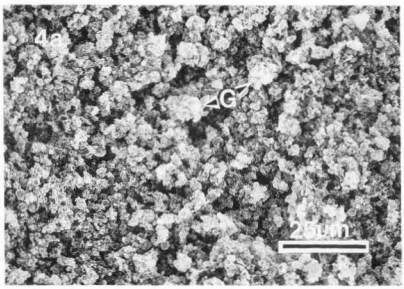
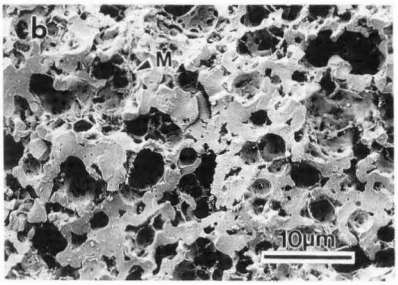


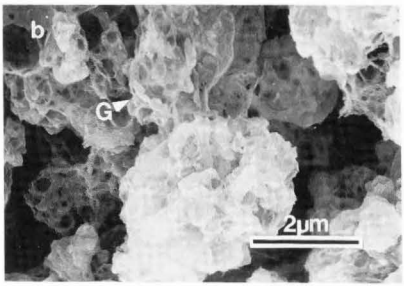
Figure 1 (at left). Mozzarella cheese after cooking/stretching but before brining. **Figure 1a**: A surface cut with a razor blade, blade marks (arrowheads) and displaced material (DM) are evident. **Figure 1b**: Cryofracturing reveals the internal structure of the protein matrix including minute pores (arrowheads) and bacteria (B). **Figure 1c**: Internal void within the cheese matrix exposed by cryofracturing; protein strands vary in thickness from 1 μm to 10 μm; bacteria (B), particulate material on protein surface (arrowheads), and salt crystals (C) are evident. The three figures are at identical magnifications.

Figure 2 (at right). Swiss cheese curd at cutting, fixed in 2% glutaraldehyde. **Figure 2a**: Cryofracturing in 2% glutaraldehyde; long voids (arrowheads) are artifacts caused by ice damage during freezing. **Figure 2b**: Cryofracturing in 50% DMSO, voids are predominantly round, with some elongated ones. **Figure 2c**: Cryofracturing in 70% ethanol, gel appears to lack diagonal voids. The three figures are at identical magnifications.









Figures 3a and b. Swiss cheese curd at a time it was pumped from the cheese vat to the pressing vat. **Figure 3b** shows the central regional of Figure **3a** at a higher magnification where the residual fat globule membrane (M) is readily observed.

Figures 4a and b. Commercial cream cheese. The fat (65% of the solids) has been removed, yielding an image of collapsed fat globule membranes (G). Figure 4b shows an enlarged representative area from Figure 4a.

surface. This material probably consists of soluble proteins and salts precipitated into the protein fibers during processing.

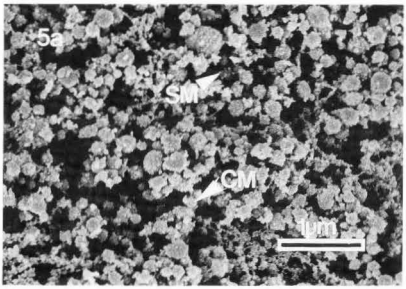
Most of the specimens cryofractured in 2% glutaraldehyde did not appear to have been damaged by ice crystals (Fig. 1b). Ice crystal damage was apparent only in the high-moisture specimens, such as yogurt (~85% water) and cheese curd (~88% water) at the early stages of cheese making (Fig. 2a). Cryoprotection with 50% DMSO, which is a common cryoprotectant for cellular material [13], reduced ice crystal damage, but some elongated openings were observed in the gel (Fig. 2b). There was no apparent ice crystal damage when the cheese products were cryofractured in 70% ethanol, and no elongated voids were seen (Fig. 2c). Void spaces within the casein matrix were spherical, showing that they were formed by fat droplets interrupting the aggregation of casein micelles. It would appear that, in general, there was only one fat droplet per void space. The casein micelles thus surrounded each fat droplet separating it from the others.

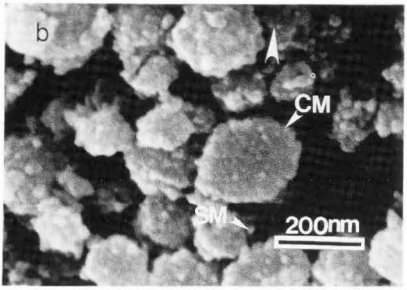
Defatting

Defatting with Freon 113 overnight removed the fat from all samples. Some residual fat globule membranes were apparent as lacy material in the void spaces which originally contained the fat droplets (Fig. 3).

Metal impregnation

As shown in Figure 3, cryofracturing produced planar surfaces with good surface information. There was minimal charging and thermal drift in the hard cheeses with lower water content which had large flat cryofractured surfaces (Figs. 3a and 3b). Highly porous specimens consistently required more metal coating (4 nm) due to charging and thermal drift. The method substantially reduced edge effect brightness and translucency of surfaces in all specimens.





Figures 5a and 5b. Yogurt. Acidified casein micelles (CM) and submicellar material (SM) 2-10 nm in diameter are evident. At higher magnification (**Figure 5b**), three nm resolution is measurable between two submicellar particles (arrowhead).

Specimen mounting and coating

Cheese samples were held securely with the finger-nail polish. With the yogurt and cream cheese samples, silver paint was used as an overcoating to establish a good electrical path from the cryofractured surface to the aluminum stub, which reduced charging (Figs. 4 and 5a). A 2 to 4 nm layer of iridium was sufficient to eliminate charging and produce a substantial secondary electron emission from all specimens.

Field emission scanning electron microscopy

Low to moderate magnification (20,000x or less) imaging was optimal at an accelerating voltage of 2 kV, with a specimen current of 0.01 nA, a condenser setting of 10, and a 15 mm working distance on the S-4000T. The resulting images had enhanced surface information throughout the entire grey value range. Charging, sample drift, sample translucency, and edge effect brightness caused no problems (Figs. 6a and 6b). Surface

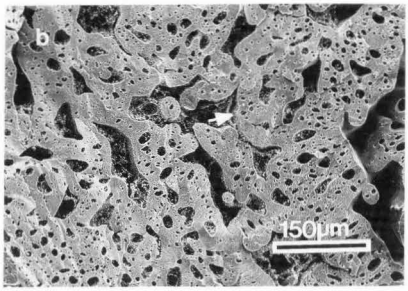


Figure 6. Mozzarella cheese, one day old. **Figure 6a**: Longitudinal (along the axis of protein fiber) cryofracture through cheese. Numerous small circular depressions, layered between narrow protein fibers are evident (arrowhead). **Figure 6b**: Cross-sectional cryofracture through cheese (90° to Fig. 6a). Protein fibers appear much larger since Figure 6a shows cheese cryofractured through the larger fibers in Figure 6b. Note the small circular depressions in a protein fiber (arrowhead). Both figures are at identical magnifications.

information was enhanced by an increased emission of secondary electrons, termed SE1 electrons, primarily from the specimens outer surfaces. Metal impregnation, iridium coating, and low accelerating voltage aid in SE1 production.

When longitudinally cryofractured Mozzarella cheese (along the axis of its fibers) was imaged, micrographs were obtained with high image detail and showed numerous small, circular depressions layered between what appeared to be narrow protein fibers (Fig. 6a). When a cross-sectional cryofracture was made of the same sample, a very different interpretation became possible (Fig. 6b), since large void filled protein fibers were seen with the internal structure between the protein

fibers exposed. The surface of fractured protein fibers appeared smooth in contrast to the numerous depressions in their exteriors. The void spaces also contained remnants of fat globule membranes. Cryofracturing in the longitudinal plane did not occur around the large fibers seen in Figure 6b, but through them. This indicates that the small voids have aligned parallel to the long axis of the protein fibers throughout the cheese.

High-resolution imaging was optimal at an accelerating voltage of 5 kV, with a specimen current of 0.008 nA, a condenser setting of 14, and a working distance of 5 mm. The resulting images were highly stable with good signal-to-noise ratios and a measurable resolution of 3 nm, allowing image recording at 100,000x (Fig. 5b).

Discussion

Milk products, such as cheese and yogurt, have been difficult to image in the SEM due to a variety of artifacts [7]. Artifacts may arise at any preparatory step, including sampling, fixation, dehydration, critical-point drying or freeze drying, mounting, metal coating, and microscopical examination.

We collected the hard cheese samples using traditional methods [3, 4, 6, 8, 12], but the yogurt and cream cheese samples were cut larger than normal. Smaller pieces would not have withstood the agitation involved in their preparation, but their porosity facilitated chemical penetration throughout the samples. The interiors of all fully prepared samples were blackened by osmium, which was indicative of complete fixation.

There were no visible differences in the hard cheese samples prepared using the three cryofracturing protocols. The low water content of cheese samples, compared to yogurt, in combination with their high protein and solute levels, allowed for fracturing under various conditions. However, the yogurt and cheese curd, which had a higher water content and larger sample size, required cryoprotection. Seventy percent ethanol appears to be suitable because it produced no appreciable elongated openings in the sample matrices. We chose Freon 22 for quick freezing of the samples because it can be used conveniently and safely. Liquified propane would also work well but involves the risk of fire and explosion [10]. Slushy nitrogen is an alternative to the environmentally damaging Freon 22 but we did not have the necessary apparatus to use this cryogen.

As a defatting agent we chose Freon 113, which worked exceptionally well, because it poses less of a health hazard than chloroform, which has a Material Safety Data Sheet (MSDS) rating of 3 (severe, cancer causing). Samples have been stored in Freon 113 for long periods with no observed detrimental effects.

The combination of metal impregnation, ultra-fine iridium metal coating, and low-voltage high-resolution FESEM yields images of cheese and yogurt of excellent image quality. The low-magnification images clearly demonstrated the distribution of milk protein and the interaction with the other milk constituents. The high-magnification images had a measurable resolution of 3 nm, close to the 2.0 to 2.5 nm resolution limits of ultra-thin sectioned biological material (Youssef, personal communication), as well as the factory-specified resolution of 2.2 nm at 5 kV of the Hitachi S-4000T (Roth, personal communication).

At a magnification of 100,000x, the protein structure is still discernable at about 3 nm. Carlino (personal communication) found no discernable structure in iridium films at that magnification. The measured thickness of iridium (4 nm) on the yogurt samples would supposedly cover this fine detail, but the sample surface was not completely flat and the actual coating would therefore be thinner than measured. Using lead staining [5] in conjunction with the Ta-F-O procedure or repeating the tannic acid-osmium-iron step one or more times might impart better electrical and thermal conductivities to the samples, allowing a thinner iridium film. Under most circumstances, however, the additional expense and labor probably are unnecessary.

The ideal accelerating voltage and emission current will vary for different microscopes. The Hitachi S-4000T at an accelerating voltage of 5 kV and a specimen current of 0.01 nA, has minimal primary electron penetration and retains very high resolution (2.2 nm).

Conclusion

The following is a summary of the protocol which should yield satisfactory results for a wide variety of milk product samples.

1A. Cut a strip of firm milk product (cheese, etc.) into 1 x 1 x 10 mm strips and transfer into vials with 2% aqueous glutaraldehyde or in appropriate buffer at room temperature for 1 hour. **or**

1B. Scoop the soft milk product up with a plastic straw (~ 1 cm in diameter) cut in half and transfer to a petri dish containing 2% glutaraldehyde. Allow to firm, cut into slabs 3 x 3 x 10 mm. Fix for 1 hour. Transfer carefully into vials with 2% aqueous glutaraldehyde or in appropriate buffer at room temperature.

Samples may be stored up to ten days at 3°C.

2. Dehydrate step-wise to 70% ethanol, through 30%, 50%, 70%, 10 minutes each.

3. Plunge sample into Freon 22 cooled with liquid nitrogen contained in a double walled vessel.

4. Transfer to liquid nitrogen and fracture perpendicular to long axis by holding with precooled insulated forceps and pressing the sample against the side of the container.

5. Remove from liquid nitrogen and return to 70% ethanol.

6. Continue step-wise dehydration to 100% ethanol through 95%, 100%, 100%, 100% ethanol, 10 minutes each.

7. Defat the sample by making step-wise

transfers in Freon 113 concentrations of 25%, 50%, 75% Freon in ethanol, then 100%, 100% Freon, 10 minutes each. Store overnight at 3°C.

8. Completely rehydrate the samples by reversing steps 7, 6 and 2.

9. Wash in a 0.1 M sodium cacodylate buffer, pH 7.2, for 5 minutes.

10. Metal-impregnate in O-F solution: {1% OsO_4 and 1.5% $K_4Fe(CN)_6 \cdot 3H_2O$ in 0.1 M sodium cacodylate buffer, pH 7.2 (O-F)} for 2 hours. The O-F solution should be discarded after 12 hours.

11. Replace O-F with 2% tannic acid in 0.1 M cacodylate buffer, pH 7.2, for 3 hours.

12. Replace tannic acid with O-F for 4 hours.

13. Replace O-F with 1% hydroquinone in distilled water for 18 hours.

14. Wash in distilled water, four times for 10 minutes each.

15. Dehydrate in ethanol step-wise, 30%, 50%, 70%, 95%, 100%, 100%, 100%.

16. Transfer to Freon 113 making step-wise transfers in Freon 113 concentrations of 25%, 50%, 75% in Ethanol, then 100%, 100%, 100% Freon 113, 10 minutes each.

17. Critical-point dry in Freon 13.

18. Mount to stub with finger-nail polish (Cutex cream enamel colorless .07).

19. Ion beam sputter coat, 2 nm.

Acknowledgements

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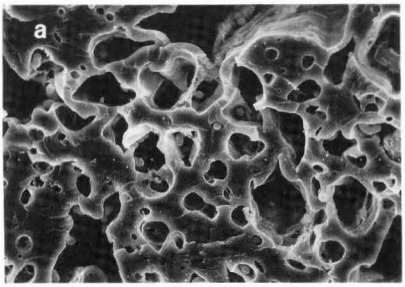
Discussions with Reviewers

M. Kalab: Cryofracturing was done using Freon 22 and Freon 113 was used for defatting. What is the reason for using these two different Freons? They are mentioned in the **Discussion** but it is not clear why Freon 113 could not be used for cryofracturing. Is Freon 113 a markedly better lipid solvent than Freon 22?

Authors: Freon 22 has been traditionally used in our laboratory for freeze fracturing. Freon 113 has been used in our laboratory for critical-point drying. From our personal experience with these two Freons, Freon 22 has performed well for cryofracturing and Freon 113 has been a very good fat solubilizer. We have no experience using them otherwise.

M. Kalab: What was the reason for using clear nail-polish with an overcoat of colloidal silver liquid? A single agent (colloidal silver paint) would reduce the time needed to mount the samples.



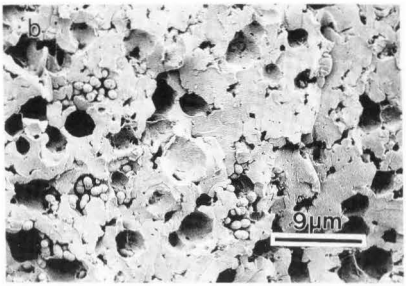


Figure 7. Mozarella cheese samples prepared with glutaraldehyde fixation only (**Figure 7a**) and with TA-F-O procedure (**Figure 7b**).

Authors: Colloidal silver paint may or may not be needed, dependent on the sample. It is a poor adhesive and samples may be easily damaged and lost when it is used alone.

B.L. Armbruster: One drawback of the OTO method is the decoration of sample surfaces with 3-4 nm precipitates. How do the authors know that the 3 nm structures in their Ta-F-O preparations are proteins and not precipitates?
Authors: In the OTO method, the highly reductive osmium is bonded with thiocarbohydrazide on and near the surface of the specimen. Ferrocyanide-osmium is less reductive (valency of VI, IV, versus VIII in osmic acid) and penetrates into the sample more rapidly, thus leading to deeper metal impregnation. Hirano *et al.* [5] have shown no surface precipitants on images up to 200,000x. Using the same procedure on non-dairy products in our laboratory also yielded a smooth surface, it is therefore presumed that the small discernable material on the surface of the dairy products is not a precipitate from the ferrocyanide-osmium.

B.L. Armbruster: Is the Ta-F-O reaction influenced by the specific buffer system used?

Improved preservation of lipids can be achieved by the addition of potassium ferricyanide to fixatives. Have the authors examined samples processed by the Ta-F-O technique which have not been defatted?
Authors: These questions are presently under investigation, but at this time are unresolved.

M. Rosenberg: Could the authors include, at least for one of the products, micrographs prepared according to a "conventional method"?
Authors: Figures 7a and 7b demonstrate the differences between a "conventional" and the TA-F-O procedure. The "conventional" image, Figure 7a, shows edge effect brightness and a lack of surface detail; voids in the matrix are very dark with little or no information. The TA-F-O image, Figure 7b, shows much higher surface detail with good information into the voids. Bacterial cell wall structure is seen.

M. Rosenberg: It seems that the new method introduces some artifacts: crystals as well as small deposits on the protein matrix. The latter is also observed in Figures 1b, 3a, and 3b. Have the authors tried to identify the nature of these bodies or to change the procedure to limit this formation?
Authors: In our early work, surface contamination had occurred occasionally. This was resolved by replacing the old source of potassium ferrocyanide with a new one from Fisher Scientific Co., Fair Lawn, NJ. The origin of the crystalline material is unresolved at this time. Their occurrence in Figure 1c would allow them to be identified as possible artifacts if they occur in subsequent applications of this procedure. The more granular nature of the surface is not due to fixation deposits on the protein matrix, but rather due to a true image of the cryofractured surface and void areas of the milk protein. Please notice in Figure 1b that the granular material is rougher and larger on the unfractured void areas.

L.J. Kiely: The described method seems exhaustive and for reasons of time and equipment (field emission are not readily available) is unlikely to find wide use.
Authors: Although maximum benefit of this procedure is seen in FESEM, it yields excellent results for non-field-emission SEM also.

L.J. Kiely: Figure 6a is of a longitudinally cryofractured surface of Mozzarella cheese and the protein strands appear to be pock-marked with numerous microcavities particularly at the edges. What is the nature of these cavities?
Authors: These are indentations caused by fat droplets, which the procedure has extracted, as we have shown previously [9].